Metabolomics Reveals Ecological Significance of Secondary Metabolites in Scutellaria baicalensis under drought stress.

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Research article

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Abstract

Background: Plant have to face the more environmental stress than animal which can dodge unfavourable circumstances by moving about, it is inevitable that the reactive oxygen species(ROS), as a product of stress, are massive generated. Excessive ROS with a powerful oxidizability under stress could do harm to the protein, including enzymes. So it is impossible to eliminated too much ROS only by antioxidant enzymes. Plant have evolved a secondary metabolism as peculiar additional pathway, but the biosynthesis of secondary metabolites would be extremely costly due to the consumption of much material and energy during suitable condition, secondary metabolism are augmented only after the stress befall, meaning the secondary metabolites vary according to the ecological environment.

Results: Using UHPLC -ESI-Q-TOF-MS/MS analysis, a total of 24 differential compounds in root of *Scutellaria baicalensis* were identified between the drought and suitable condition. Based on Results of t-test analysis（P＜0.05）between various groups, ions whose VIP value ≧2, the most significant differential chemical markers of the drought condition were citric acid, shikimic acid, baicalin, wogonoside, baicalein, wogonin, 3,5,7,2',6'-pentahydroxyflavanone, 5,2',6'-trihydroxy-7,8 -dimethoxyflavone, chrysin, eriodictyol, 5,8-dihydroxy-6,7-dimethoxy flavone, highlighting that most of them were free flavonoids with many phenolic hydroxyl group of flavonoids, with a characteristic of higher avtivities.

Conclusions: The diversity of secondary metabolites plays a crucial role, *S. baicalensis* modified the ability to eliminate ROS and matained the equilibrium of ROS through the biosynthesis and convertion between these flavonoid which contain many compounds, like an intricate buffer solution.

Background

The animal subsist by moving to dodge unfavourable circumstances, but the plant have to face the various adversity such as the high-temperature, drought, low soil fertility, et al. Under the unfavourable condition the light energy which the chlorplasts absorbed is incompatible with that of capture CO_2, leading to overcapacity of light energy, besides, the closed stomata arising from the increased abscisic acid under adversity blocked O_2 emissions outward, and was reduced to $O^{\bullet-}_2$(Mehler reation) [1]. $O^{\bullet-}_2$ can be convered into ·OH, H_2O_2. They are named as reactive oxygen species(ROS) due to their powerful oxidation. ROS can modify the structures of protein, including enzymes, by affecting the disulfide bridges, and regulate various metabolism. Suitable level of ROS can act as messengers and regulate various physiological responses in plants [2-3], but once ROS is over-produced, the redundant ROS can alter adjacent molecular configuration, and lead to reduce the cell-membrane stability, DNA strand distruction, protein crosslinking, peptide chains break, et al, with a result of metabolic disorder, even cell death [4-5]. It have been confirmed that increased ROS is a result of unfavourable circumstances [6-7] , $O^{\bullet-}_2$ with 3 fold increase, H_2O_2 with 10 fold increase under certain condition [8-9]. Because of comparatively stable and weaker oxidation, H_2O_2 can be long-distance transported and become as a signal to regulate metabolism[10-11].

ROS resulting from unfavourable circumstances are eliminated mainly by antioxidant enzyme, including superoxide dismutase(SOD), catalase(CAT), peroxidase (POD), et al, and secondary metabolites, including carotenoids, tocopherols, and phenolics et al. $O^{\cdot -}_2$ is dismutated to H_2O_2 either spontaneously or by SOD, and then dismutated into H_2O_2 and O_2 by CAT or POD. The ROS would not do harm to them under suitable level. However, the antioxidant enzyme are also protein, SOD with 2 subunits, CAT with 4 subunits, their some -SH groups which maintain the secondary structure and tertiary structure are liable to be injured if the plant be in severe abiotic stresses [12], therefore, the plant can not survive only by the antioxidant enzymes, the secondary metabolism are indispensable.

The secondary metabolites in a plant are numerous, by Ultra-High-Performance Liquid Chromatography a total of 447 metabolites in *Isatis indigotica* Fortune, 128 in American ginseng root, 132 in *Scutellariabaicalensis*, 122 in *Moringa oleifera* leaves were identified [13-16], most of them are secondary metabolites. Their activities differ from each other, their proportion varied according to the changing environmental conditions[17]. Why do plant contains so many secondary metabolites? What is the connection between them? Drought is one of the most severe abiotic stresses in plant growth and development. *Scutellaria baicalensis* Georgi distributed throughout semi-arid steppe, its root is rich in various flavonoids with diverse structure[18], and have anti-inflammatory, anti-tumor, and anti-HIV activities[19]. Drought is a main factor affecting the flavonoids in *S. baicalensis*[20]. At adverse environmental conditions, plants produce various kinds of primary and secondary metabolites to protect themselves.

Metabolomics, an untargeted biochemical approach to monitor the metabolites, is a research field used to acquire comprehensive information on the varied metabolites. Quantitative plant metabolomics, can improve our understanding of plant biochemistry and metabolism under both normal and stress conditions [21-22], has been considered as the most promising approaches for the detection of primary and secondary metabolites in abiotic stresses [23-24]. Here, based on the difference before and after the stress we investigated the biological significance of flavonoids in *S. baicalensis* under water deficit conditions.

Results

Identification of Chemical Markers in Radix Scutelariae

MassLynx V4.1was employed for the analysis of the chemical constituents of Radix Scutelariae. The chemical composition was elucidated by the spectral information obtained from secondary ion mass spectrometry, which was cross-referenced with the retention time, mass-to-charge ratio, molecular weight, structural formula and elemental composition of known ingredients in Radix Scutelariae.On the basis of the VIP results, the candidate ions between the slight drought, the severe drought, and the control were identified tentatively.

When taking the identification of baicalin as an example, in positive mode, the ion (RT =4.92 min and $[M+H]^+$ = 447.12) detected in the slight drought -treated sample was calculated to be $C_{21}H_{19}O_{11}$ based on

the elemental composition, fractional isotope abundance, and Chemspider database information. The main MS/MS fragment ions of peak 9 were *m/z* 271 and *m/z* 253, indicating that the fragments may be $C_{15}H_{11}O_5^-$ and $C_{15}H_9O_4^-$, which could indicate the loss of glucuronic acid (176 Da) and H_2O_2 (25 Da), respectively. With this integrated information, the ion was finally confirmed to be that of baicalin (Fig. 1).The corresponding mass spectrums and related structures were shown in Fig.1. According to the above-mentioned analytical method, a total of 24 chemical markers that were differentially expressed between the two Radix Scutelariae treatment groups were successfully identified, including 18 candidate ions in positive ion mode and 6 candidate ions in negative mode. Using Waters Masslynx software, we finally confirmed their identities with MS/MS data. UPLC-HDMS chromatograms of Scutellaria root in positive ion mode and in negative ion mode are shown in Fig. 2. Detailed information on the identified components is shown in Table 1.

Characteristic Multivariate Metabolomic Data Analysis

The PCA model was used to identify the difference in metabolites between the slight drought, the severe drought, and the control groups. The PCA score plots are shown in Fig.3. OPLS-DA was used to discriminate between the groups as well. As shown in Fig. 4, the group difference were clearly divided into three regions, indicating that there were significant chemical differences between them, and the established metabolomics method could successfully characterize the chemical characteristics. VIP values are commonly used to evaluate the contribution of variables in OPLS-DA. Based on the results of the t-test (P□0.05) between groups, ions with VIP values ≥2 were selected and regarded as the most significant differential chemical markers between the severe drought and the control. With this, Shikimic Acid, Citric acid, 3,5,7,2',6'-Pentahydroxyflavanone, Eriodictyol, Baicalin, Wogonoside, 5,2',6'-Trihydroxy -7,8- dimethoxyflavone, Baicalein, Wogonin, Chrysin, 5,8-Dihydroxy-6,7- dimethoxyflavone presented a significant differences. As shown in Fig. 6.

Discussion

Drought increased secondary metabolism

In this paper, only two primary metabolites were observed in the mass spectral data.Citric acid, a major substance in the tricarboxylic acid cycle, decreased remarkably to the continuous, incremental drought, indicating that the primary metabolism was weaked. The shikimic acid from which various flavonoids originated is a branch point of the primary and the secondary metabolic pathways , the decreased citric acid content and the increased shikimic acid content indicating that the secondary metabolism was enhanced, with a result of increasing secondary metabolites, a total of 9 secondary motabolites being all increased under the slight drought.

The shikimic acid located upstream of the citric acid, the Fig. 5 showed the shikimic acid are almost equal the severe drought, besides, the citric acid in the severe drouht decreases heavily, which mean that more shikimic acid should be tranformed into secondary metabolites. But except for 5,2',6'-Trihydroxy-7,8-

dimethoxyflavone, all the severe drought below the slight drought, which may be due to excessive drought, it was very probable that the *S. baicalensis* produce more ROS under the severe drought, some secondary metabolites react with ROS and be consumed [25-26].

Biological significance of varied compounds

Two features of these increased secondary metabolites invited our special attention. First. the molecular structure of the secondary metabolites dictated the biological effect by the number and sites of the phenolic hydroxyl groups in flavonoids. The molecular structure diagram of the flavonoids was shown in Fig. 7. It has been proved that the number of hydroxyl groups on the B ring directly impact the activity, which is also markedly enhanced when a double bond is introduced into the C ring [27]. The hydroxyl group at positions C-5 and C-7 together in the A ring, as well as the C-3', C-4' and C-5' sites, on the B ring can all increase the activities obviously [28]; , in another position such as C-6 can also increase the activity [29-30], another study showed that baicalein is more than 7 times more bioavailable than baicalin due to hydrophilic variations [31]. Secondly, except for the baicalin and the wogonoside, the other flavonoids were free flavonoids, not a saccharides derivatives. The composite enzymes of flavonoids biosynthesis located at endoplasmic reticulum, it is difficult for flavonoid glycosides to permeate freely into and out of the biomembrane with phospholipid bilayer due to hydrophilic saccharides. The biomembranes of animals and plants are the same; it has been proven that baicalein is 2~5 times more antibiotically active than baicalin and 1~3 times better at inhibiting IL-1β converting enzymes [31], a study showed that the activities of flavanone disappear when a sugar moiety is introduced into the A ring [27]., Flavonoid glycosides therefore are regarded as superfluous flavonoids; when required, they work mainly after conversion into free flavonoids [26]. It's very interesting that above mentioned secondary metabolites are all higher active, and lower contents, meaning the effect of compositions with a lower content can not be ignored.

ROS increases rapidly under stress. Whether overabundance or shortage, is harmful, depends on the delicate equilibrium between production and scavenging [12], maintained by antioxidant. Under severe stress, the secondary metabolites would highlight, be coordinated with the ROS. The more ROS was produced, the higher the flavonoids activity. The regulation of flavonoids activities were performed through the biosynthesis and interconvertion between these flavonoid. For one case of baicalein or wogonin, if the ROS became higher, the chrysin only with C-5 and C-7 hydroxylation could also be added a hydroxyl or methoxy group, as well as baicalin and wogonoside were removed a glucuronic acid by baicalein7-O-glucuronosyl transferase or β-glucuronidase, and converted rapidly into the baicalein or wogonin with higher activities [32],, and vice versa. From the Fig.5 such pathway was probably numerous.

Conclusions

The diversity of secondary metabolites plays a crucial role. A variety of secondary metabolites in *S. baicalensis* assembled into a complex with flexible proportion, modified the ability to eliminate redundant

ROS and matained the equilibrium of ROS through the biosynthesis and convertion between these flavonoid, like an intricate buffer solution as quickly, delicately as possible.

Methods

Plant Materials and Reagents

The *Scutellaria baicalensis* Georgi, identified by Prof Xiang-cai Meng, were collected from the medical plants garden in Heilongjiang University of Chinese medicine. A total of 18 plants of 2 years were tranplanted into 3 flowerpots in May 2018, 6 plants every one. The flowerpots were slice the bottom off, then deposited underground, grew naturally without any intervention. In October(Temperature at around 10℃), pulled the flowerpots out from the earth, put the plastic sheeting under the bottom to insulate soil water, then returned to original position. The slight drought treatment: Collected the sample when topsoil is around 2cm; the severe drought treatment: Collected the samples when topsoil is around 7cm; the control: Keep the soil moist. A total of 5 plants be selected from a flowerpot, washed the dirt off, removed the xylem, freeze-dried, and then grinded into a fine powder, respectively. A voucher specimen(S20181005) was deposited at the College of Pharmacy, Heilongjiang University of Chinese Medicine, China.

Reagents: Acetonitrile, HPLC grade, was obtained from Merck (Darmstadt, Germany); methanol (HPLC grade) was purchased from Fisher Scientific Corporation (Loughborough, UK); ultrapure water was produced by a Milli-Q Ultra-pure water system (Millipore Corporation, MA, USA); Leucine-enkephalin was purchased from SIGMA Corporation (USA). All other reagents were of analytical grade.

Preparation of Extraction for UHPLC-ESI-Q-TOF-MS/MS Analysis

Collection and preparation of plant samples: Fine powder (150 mg) and 50 ml 70% ethyl alcohol were placed into conical flasks and ultrasonically extracted for 1 h, and the volume lost was replaced with fresh 70% ethyl alcohol. Finally, the supernatant was filtered with a 0.22 μm microporous filter for UPLC analysis.

Conditions of Analysis

Ultra-performance liquid chromatography

Chromatographic separation was performed on an ACQUITY UPLC system (Waters Corporation, Milford, MA) consisting of a binary solvent manger, a sample manager and a column compartment. The column used was an UPLC^{TM} BEH C_{25} column (100mm× 2.1mm 1.8μm, Waters Corporation, Milford, USA). Column temperature was maintained at 40 °C for all analyses, Sample warehouse temperature at 10 °C. The optimal mobile phase consisted of a linear gradient system of (A) 0.1% formic acid in acetonitrile and (B) 0.1% formic acid in water: 0 to 1.5min, 16 to 22% A; 1.5 to 5min, 22 to 30% A; 5 to 9 min, 30 to 40% A; 9 to 12min, 40 to 70% A; 12 to 15 min, 70 to 100% A. The detection wavelengths 190 to 400nm

ultraviolet full wavelength scanning. The flow rate was set to 0.4 mL/min. Injection volume was 3μL. The ultraviolet pectrophotometry of the positive and negative ions were performed through flowing straight to mass spectrometer. All the samples were kept at 4 °C during the analysis.

Mass spectrometry.

The positive ion ionization mode: the capillary voltage was 3.0 kV, the sampling cone voltage was 25 V, the extraction voltage was 4.0 V, the desolvation gas temperature was 350 °C, the desolvation gas flow was 600 L/h, the source temperature was 110 °C, and leucine enkephalin at a concentration of 0.2 ng/mL was used via a lock spray interface at a flow rate of 100 μl·min^{-1} for monitoring in positive ionization mode ([M+H]+ = 556.2771) to ensure accuracy during MS analysis. The lock spray frequency was set to 5 s, and scan averaging for correction was every 0.02 s and 0.4 s per scan. The scanning range was m/z 100~1500.

The negative ion ionization mode: the capillary voltage was 2.2 kV, the sampling cone voltage was 25 V, the extraction voltage was 3.5 V, the desolvation gas temperature was 350 °C, the desolvation gas flow was 600 L/h, the source temperature was 110 °C, and leucine enkephalin at a concentration of 0.2 ng/mL was used via a lock spray interface at a flowrate of 100 μl·min^{-1} for monitoring in negative ionization mode ([M+H]+ = 556.2771) to ensure accuracy during the MS analysis. The lock spray frequency was set to 5 s, and scan averaging for correction was every 0.02 s and 0.4 s per scan. The scanning range was m/z 100~1500.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

PCA: principal components analysis;OPLS-DA:orthogonal partial least squre -discriminate analysis; VIP: variable importance in the projection; RT: retention time.

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Table

Table 1. Characterization of compounds in fresh Scutellaria root extract by UPLC-HDMS

Peak No.	Rt (min)	Selected ion	Measured mass(m/z)	Calc.mass (m/z)	Error (ppm)	Formula	MS/MS fragment ion (m/z)	Identification
1	0.54	$[M-H]^-$	173.15	173.03	3.3	$C_7H_{11}O_5$	155,137,111	Shikimic Acid
2	0.62	$[M-H]^-$	191.14	191.15	2.9	$C_6H_7O_7$	173,147	Citric acid
3	2.17	$[M+H]^+$	305.26	305.21	4.0	$C_{15}H_{13}O_7$	287,153	3,5,7,2',6'-Pentahydroxyflavanone
		$[M-H]^-$	303.25	303.19	3.5	$C_{15}H_{11}O_7$		
4	2.8	$[M+H]^+$	549.15	549.16	1.6	$C_{26}H_{29}O_{13}$	531,513,495411,375	Aspenin-6-C-Arabinose-8-C-glucoside
		$[M-H]^-$	547.14	547.14	1.3	$C_{26}H_{27}O_{13}$		
5	3.19	$[M+H]^+$	549.15	549.16	0.9	$C_{26}H_{29}O_{13}$	531,513,495411,375	Chrysin-6-C-glucose-8-C-Araboside
		$[M-H]^-$	547.14	547.14	2.7	$C_{26}H_{27}O_{13}$		
6	4.16	$[M+H]^+$	477.10	477.10	2.1	$C_{22}H_{21}O_{12}$	301,286	5,7,2'-trihydroxy-6-methoxyflavonoids-7-O-glucuronide
		$[M+H]^-$	475.08	475.08	0.2	$C_{22}H_{19}O_{12}$		
7	4.35	$[M-H]^-$	287.26	287.19	2.6	$C_{15}H_{11}O_6$	251,135	Eriodictyol
8	4.56	$[M+H]^+$	347.07	347.07	4	$C_{17}H_{15}O_8$	332,314	5,7,2',5'- tetrahydroxy -8,6'- dimethyl oxyflavones
		$[M-H]^-$	345.05	345.06	2.6	$C_{17}H_{13}O_8$		
9	4.92	$[M+H]^+$ $[M-H]^-$	447.12	447.09	2.5	$C_{21}H_{19}O_{11}$	271,253	Baicalin
10	5.68	$[M+H]^+$	447.09	447.09	4	$C_{21}H_{19}O_{11}$	285	Oroxylin A-5-O glucoside
		$[M-H]^-$	445.07	445.07	2.2	$C_{21}H_{17}O_{11}$		
11	5.83	$[M+H]^+$	447.09	447.09	4.7	$C_{21}H_{19}O_{11}$	271	Baicalin isomers
		$[M-H]^-$	445.07	445.07	1.8	$C_{21}H_{17}O_{11}$		
12	5.95	$[M+H]^+$	477.10	477.10	4.2	$C_{22}H_{21}O_{12}$	301,286	5,7,8-trihydroxy-6-methoxyflavone-7-O-glucuronide
		$[M-H]^-$	475.08	475.08	4.4	$C_{22}H_{19}O_{12}$		
13	6.19	$[M+H]^+$	461.10	461.10	4.6	$C_{22}H_{21}O_{11}$	285,270	Wogonoside
		$[M-H]^-$	459.09	459.09	3	$C_{22}H_{19}O_{11}$		
14	6.2	$[M-H]^-$	429.08	429.08	3.5	$C_{21}H_{17}O_{10}$	253	Chrysin -7-O-glucuronide
15	6.44	$[M+H]^+$	477.10	477.10	2.9	$C_{22}H_{21}O_{12}$	301	5,6,7-trihydroxy-8-methoxyflavone-7-O-glucuronide
		$[M-H]^-$	475.08	475.08	3.2	$C_{22}H_{19}O_{12}$		
16	7.01	$[M+H]^+$	315.30	315.22	1.1	$C_{17}H_{15}O_6$	282,285	5,8-dihydroxy-6,7-dimethoxyflavones
17	7.05	$[M-H]^-$	489.09	489.10	0.8	$C_{23}H_{21}O_{12}$	313,298	5,7-dihydroxy-6,8-dimethoxyflavone-7-O-glucuronide
18	7.97	$[M-H]^-$	329.88	329.85	3.5	$C_{17}H_{13}O_7$	299	5,2',6'-Trihydroxy-7,8-dimethoxyflavone
19	8.21	$[M+H]^+$	271.05	271.06	3.6	$C_{15}H_{11}O_5$	253,241	Baicalein
		$[M-H]^-$	269.04	269.04	1.9	$C_{15}H_9O_5$		
20	10.51	$[M+H]^+$	285.05	285.07	4.6	$C_{16}H_{13}O_5$	270	Wogonin
		$[M-H]^-$	283.04	283.06	4.2	$C_{16}H_{11}O_5$		
21	10.67	$[M+H]^+$	255.06	255.06	4.5	$C_{15}H_{11}O_4$	209	Chrysin
		$[M-H]^-$	253.04	253.05	3.5	$C_{15}H_9O_4$		
22	11.01	$[M+H]^+$	375.10	375.18	4.3	$C_{19}H_{19}O_8$	360,345,327	Skullcapflavone
23	11.05	$[M+H]^+$	375	375	1.1	$C_{19}H_{19}O_8$	345	5,7-dihydroxy-6,8,2',3'-tetramethoxyflavones
		[M-H]-	373	373	2.9			

						$C_{19}H_{17}O_8$		
24	11.45	$[M+H]^+$	345.0	345.09	4.3	$C_{25}H_{17}O_7$	330,315	5,2-dihydroxy-6,7,8-trimethoxyflavones
		$[M-H]^-$	343.07	343.08	1.9	$C_{25}H_{15}O_7$		

Figures

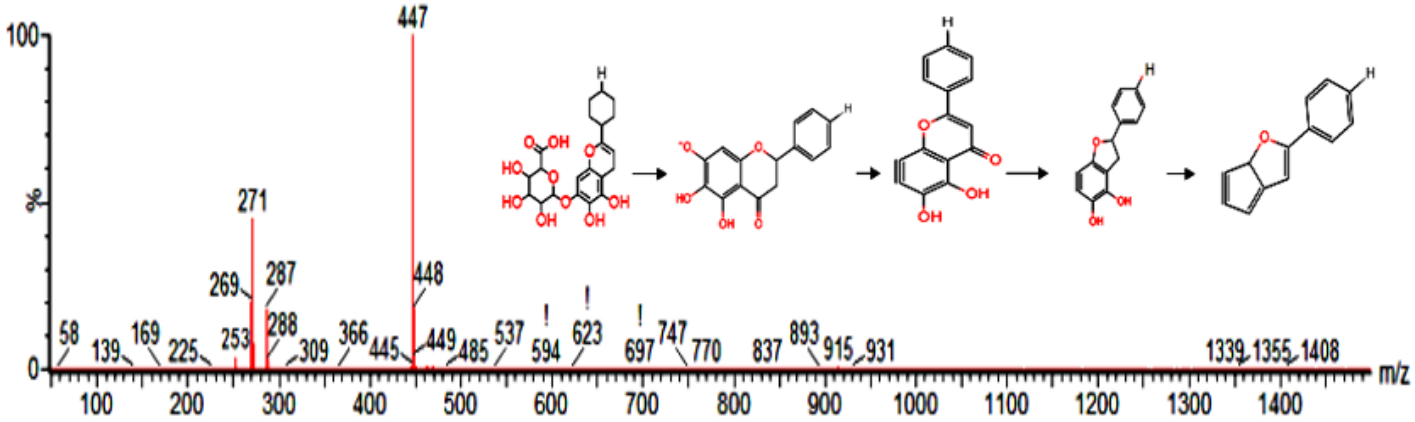

Figure 1

MS-MS and the proposed framentation pathway of baicalin

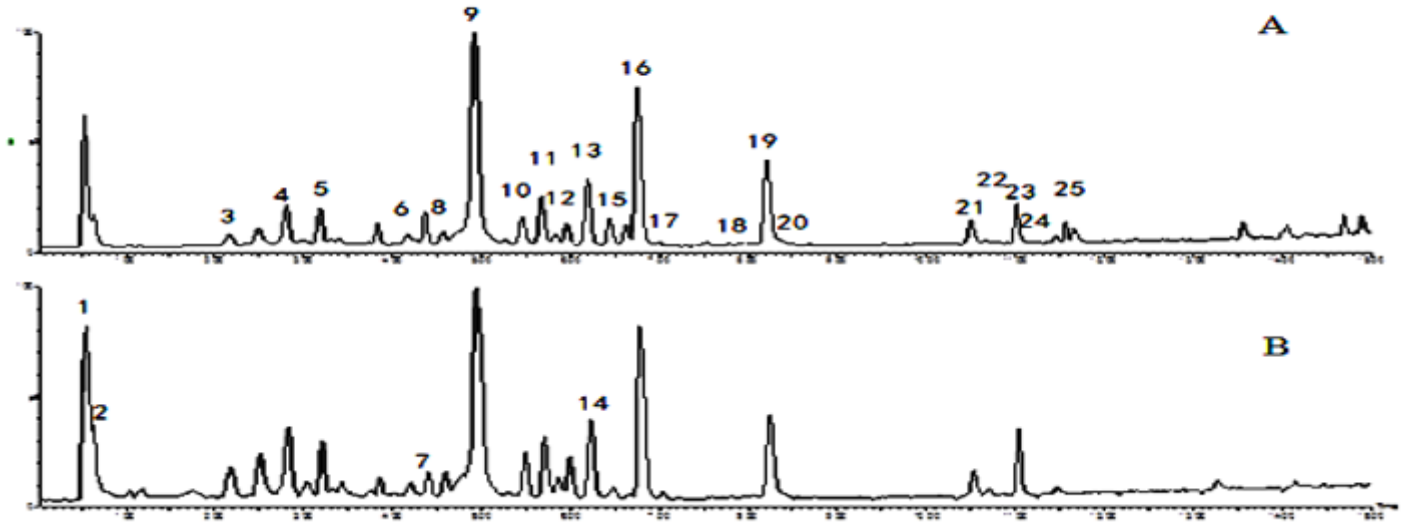

Figure 2

Chromatograms of Scutellaria root in positive ion mode and in negative ion mode. (A)positive ion mode ; (B) negative ion mode.

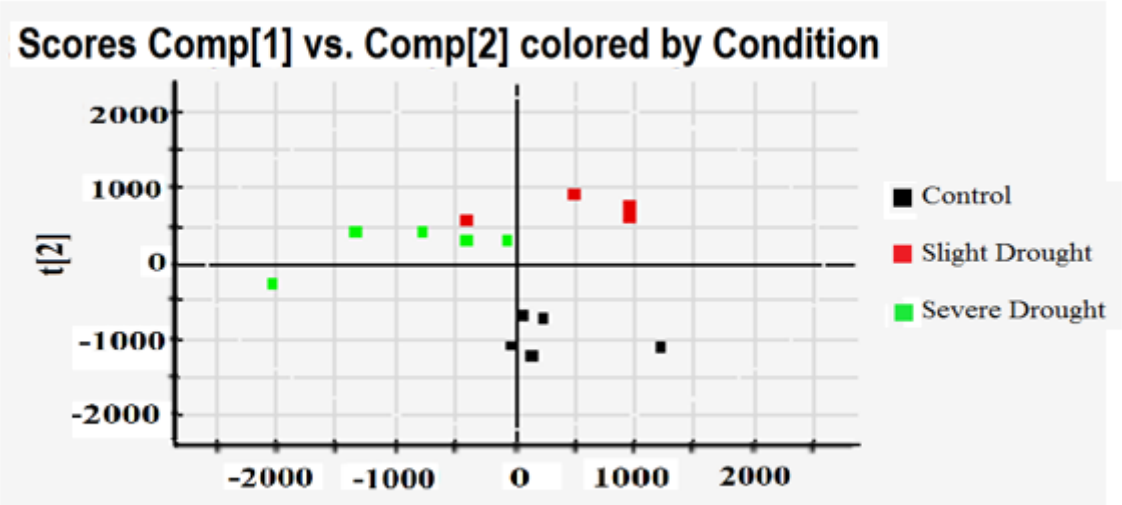

Figure 3

PCA score diagram of UPLC-Q/TOF-MS data PCA analysis in positive ion mode

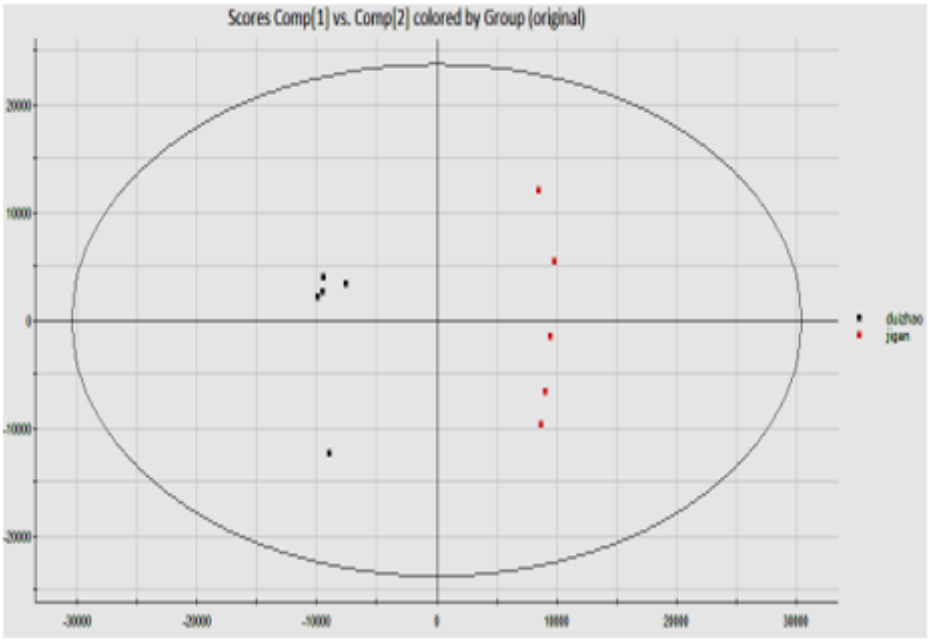

Figure 4

OPLS-DA analysis between severe drought treatment and control in positive ion mode.

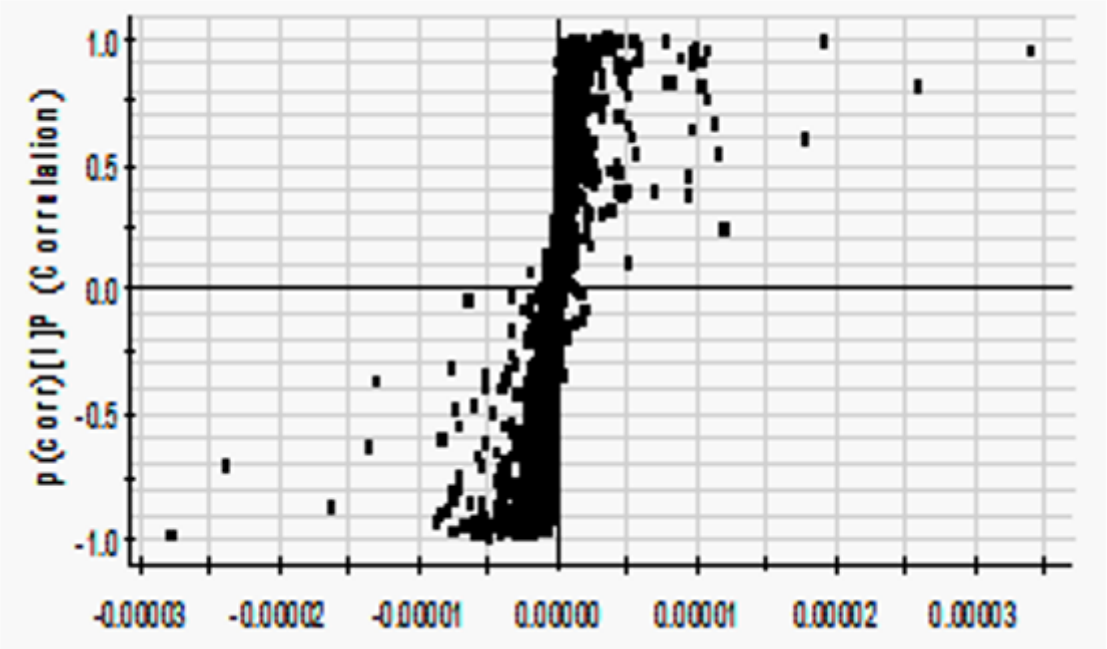

Figure 5

S-plot of the OPLS-DA analysis between severe drought and control in positive ion mode. Most of varied compounds were secondary metabolites with polyphenolic hydroxyl group, without a sugar moiety, showing increased activities. A: Citric acid; B: Shikimic Acid; C: Baicalin; D: Wogonoside; E: Baicalein; F: Wogonin; G: 3,5,7,2',6'-Pentahydroxyflavanone; H: 5,2',6'-Trihydroxy-7,8-dimethoxyflavone; I: Chrysin; J: Eriodictyol; K: 5,8-Dihydroxy-6,7-dimethoxyflavone. C: Control D1: Slight Drought D2: Svere Drought

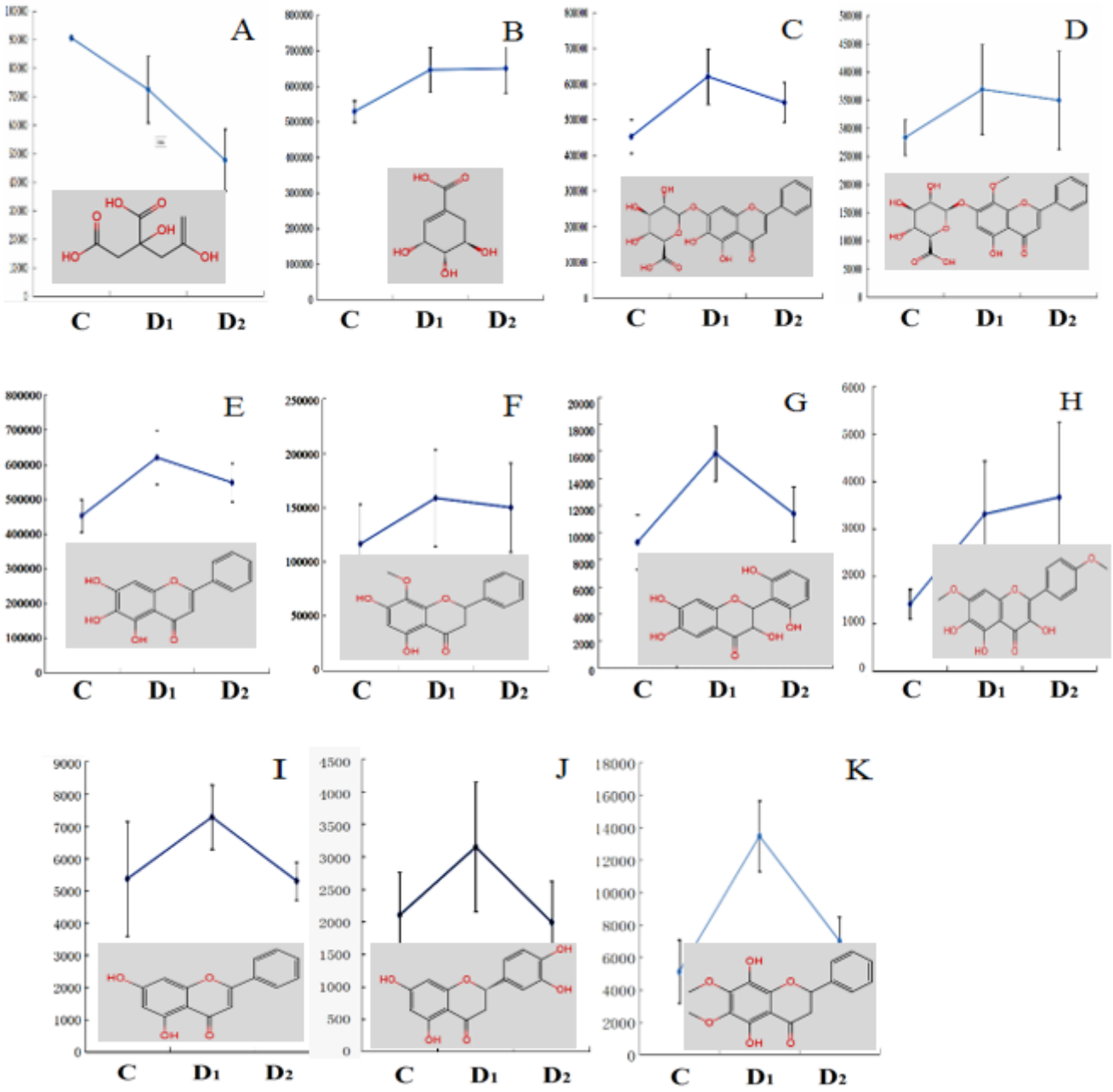

Figure 6

Change of 11 compounds under drought

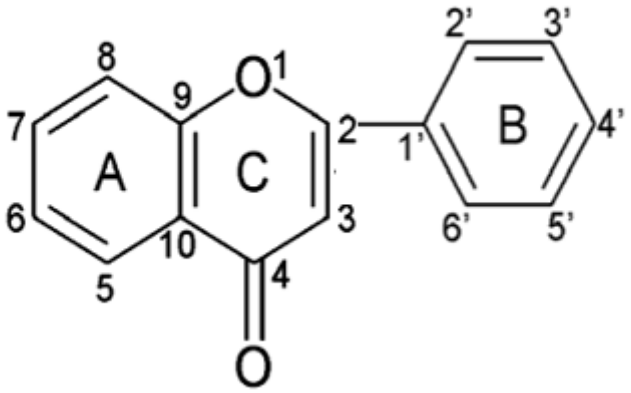

Figure 7

Molecular structure diagram of the flavonoids